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Exploring "one-shot" kinetics and small molecule analysis using the ProteOn XPR36 array biosensor

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Abstract

A ProteOn XPR36 parallel array biosensor was used to characterize the binding kinetics of a set of small molecule/enzyme interactions. Using one injection with the ProteOn's crisscrossing flow path system, we collected response data for six different concentrations of each analyte over six different target protein surfaces. This "one-shot" approach to kinetic analysis significantly improves throughput while generating high-quality data even for low-molecular-mass analytes. We found that the affinities determined for nine sulfonamide-based inhibitors of the enzyme carbonic anhydrase II were highly correlated with the values determined using isothermal titration calorimetry. We also measured the temperature dependence (from 15 to 35 °C) of the kinetics for four of the inhibitor/enzyme interactions. Our results illustrate the potential of this new parallel-processing biosensor to increase the speed of kinetic analysis in drug discovery and expand the applications of real-time protein interaction arrays.

Keywords: Surface plasmon resonance; Protein/protein interaction; Kinetics; SPR

It is evident that affinity-based biosensor technology has changed the way we view molecular interactions. The ability to effectively "see" complex formation in real time provides detailed insights into the binding process between two or more reactants. Acceptance of biosensor technology has grown considerably over the past 15 years, as is evident by more than 1000 articles that use optical biosensors being published each year now [1–3]. Throughput has become the primary bottleneck as biosensor technology migrates from the stable of analytical tools employed in protein/protein interaction analysis to the front lines of drug discovery.

Bio-Rad Laboratories' new ProteOn XPR36 array biosensor represents one approach to increasing sample throughput. The ProteOn system uses a surface plasmon resonance (SPR)[1]-based detector [4] to monitor complex formation between analytes and target molecules in real time and without labeling. A unique feature of the ProteOn technology is its use of six parallel flow channels. Although parallel processing could improve sample throughput by sixfold over serial processing systems, more important is that the ProteOn system is able to rotate the configuration of the six flow cells 90°, a capability that provides a novel method of creating a 36-spot array.

Fig. 1A illustrates how the six flow paths can be used in a "vertical" direction during the immobilization phase to prepare in parallel six lanes of target protein. Then the flow paths can be automatically rotated to the "horizontal" position (Fig. 1B), making it possible to test the binding of

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[1] *Abbreviations used:* SPR, surface plasmon resonance; DMSO, dimethyl sulfoxide; sulfo-NHS, sulfo-*N*-hydroxysuccinimide; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide.

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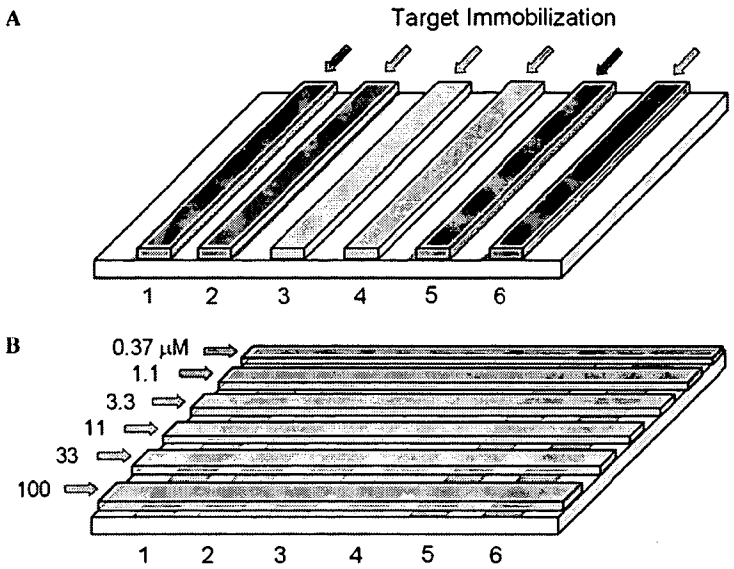

Fig. 1. Schematic of the ProteOn XPR36 flow cell design. (A) Six parallel flow paths oriented in the vertical direction for target immobilization. (B) Six parallel flow paths oriented in the horizontal direction for analyte injections of different concentrations (0.37–100 μM in a threefold dilution series).

six different analytes simultaneously. In this case, each analyte would pass over the six different target surfaces that are located within each horizontal channel. There are also reference spots located in the horizontal channel immediately before and after each reaction spot that allow for internal referencing.

The use of crisscrossing flow paths provides for a number of interesting experimental applications. One of the first applications we wanted to explore was the ability to collect kinetic data for six different concentrations of analyte at the same time. Historically, response data for different analyte samples have been collected sequentially. Parallel collection of different analyte concentrations could improve sample throughput and also render obsolete the need to regenerate the target sensor surface.

To test the throughput and performance of the ProteOn instrument, we selected a well-characterized small molecule interaction system, namely the binding of sulfonamide-based inhibitors to the enzyme carbonic anhydrase II. The enzyme was coupled at different densities within the six flow channels using amine chemistry. We found that response data collected simultaneously for six concentrations of each compound could be globally fit to a simple interaction model. The affinities determined from the ProteOn instrument correlated well with those determined using titration calorimetry and Biacore.

The application of "one-shot" kinetics and the ability of the ProteOn instrument to collect kinetic data for small molecule interactions could significantly expand the speed and application of optical biosensor technology in drug discovery.

Materials and methods

All experiments were performed using ProteOn XPR36 instruments developed by Bio-Rad Haifa (Haifa, Israel). Carbonic anhydrase isozyme II from bovine erythrocytes, nine sulfonamide inhibitors (Table 1), dimethyl sulfoxide (DMSO), sulfo-*N*-hydroxysuccinimide (sulfo-NHS), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC), ethanolamine–HCl, and buffer reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1
Compounds used in analysis

Sample	Compound	Molecular mass (Da)	Concentration (μM)
A	(±)-Sulpiride	341	250
B	Sulfanilamide	172	50
C	Furosemide	331	50
D	4-Carboxybenzenesulfonamide	201	50
E	Dansylamide	250	10
F	1,3-Benzenedisulfonamide	236	10
G	Benzenesulfonamide	157	50
H	7-Fluoro-2,1,3-benzoxadiazole-4-sulfonamide	217	2
I	Acetazolamide	222	2

Carbonic anhydrase II immobilization

The ProteOn sensor chips, which are glass prisms coated with gold and an alginate-based layer, were preconditioned with two short pulses each (10 s) of 50 mM NaOH, 100 mM HCl, and 0.5% SDS. After the system was equilibrated with PBS-T buffer (20 mM Na-phosphate, 150 mM NaCl, and 0.005% Tween 20, pH 7.4), carbonic anhydrase was immobilized at 25 °C using a flow rate of 30 µl/min. Five channels were activated for 5 min with a mixture of EDC (0.2 M) and sulfo-NHS (0.05 M). To create different immobilization densities, this mixture was diluted with water for the different channels. For example, the sulfo-NHS/EDC mixture was injected without dilution over channel 1 and then diluted 5-, 10-, 20-, and 30-fold for channels 2 to 5, respectively. Channel 6 was not activated to serve as an additional reference surface. Immediately after the surfaces were activated, carbonic anhydrase (125 µg/ml in 10 mM sodium acetate, pH 5.0) was injected across channels 1 to 5 for 5 min. Finally, channels 1 to 5 were blocked with a 5-min injection of 1 M ethanolamine–HCl (pH 8.5). An example profile of this immobilization procedure is shown in Fig. 2. This method resulted in carbonic anhydrase coupled at response levels of 9150, 7600, 6600, 4500, and 3100 RU (1 RU = 1 pg protein/mm^2) in channels 1 to 5, respectively. The standard deviation in the immobilization level from the six spots within each channel was less than 3%.

Analyte injections

Kinetic response data were collected for the nine sulfonamide inhibitors listed in Table 1. Also included in the table are the molecular mass and highest concentration of compound used in the dilution series. Each compound was tested at six concentrations using a threefold dilution series. The six concentrations of each analyte were injected simultaneously (a single injection) at a flow rate of 100 µl/min for a 1-min association phase, which was followed by a 2-min dissociation phase. Standard experiments were performed in PBS-T with 3% DMSO at 25 °C. As part of a thermodynamic study, additional response data were collected for compounds A, B, D, and F from 15 to 35 °C in incremental temperature jumps of 5 °C. A DMSO calibration curve was generated over a DMSO concentration range of 2.4 to 3.6%.

Data processing and analysis

Fig. 3 shows the raw and processed data for the injection of furosemide. Response data from the ProteOn instrument (Fig. 3A) were first zeroed on the y axis just prior to the start of the analyte injection (Fig. 3B). Responses from the reference positions before and after each reaction spot were then averaged and subtracted from the reaction spot data to correct for any bulk shifts due to a mismatch between the sample and running buffer as well as any nonspecific binding (Fig. 3C). Data from the DMSO concentration series was used to correct for excluded volume effects [5]. Finally, the data for each analyte concentration series collected over the same target density surface were globally fit to a 1:1 interaction model [6] with one local parameter for surface capacity (R_{max}), one association rate constant (k_a), and one dissociation rate constant (k_d). The ratio of the rate constants (k_d/k_a) yielded the value for the equilibrium dissociation constant (K_D).

Results

One-shot kinetics

A novel feature of the ProteOn XPR36 system is its ability to collect kinetic data for six different analyte

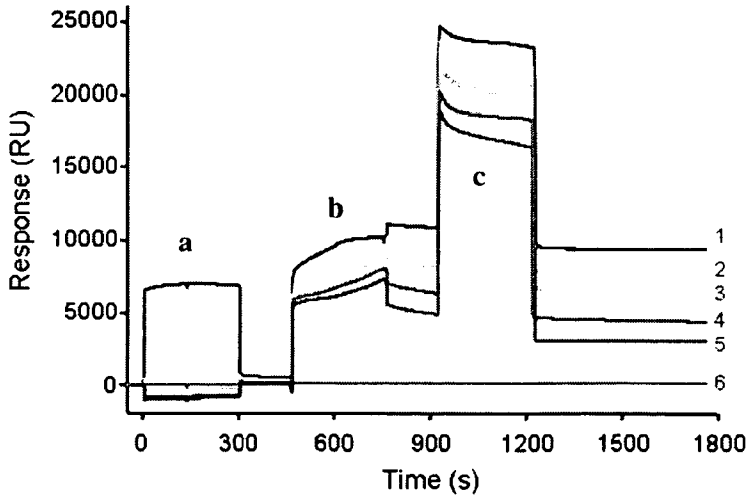

Fig. 2. Immobilization of carbonic anhydrase on the surface of a ProteOn sensor chip. The plot shows the overlay of sensorgrams for the six channels with the sulfo-NHS/EDC (a), carbonic anhydrase (b), and ethanolamine (c) injections highlighted. Individual channels are indicated by the numbers on the right-hand side of the figure.

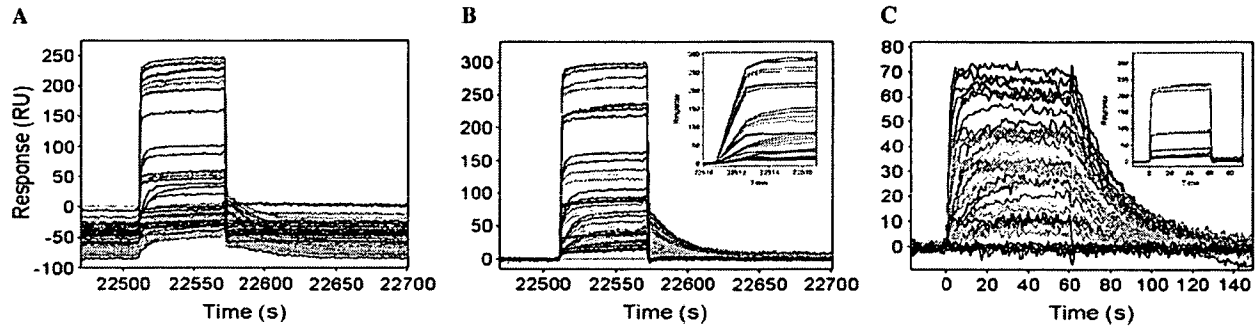

Fig. 3. Data processing. (A) Raw data for six concentrations of furosemide injected over six channels on the ProteOn chip. (B) Furosemide data after zeroing on the *y* axis. The inset shows an expanded view of the start of the injection phase. (C) Furosemide data after zeroing for the start time of the injection and subtracting out data from the internal reference spots (reference spot data are shown in the inset).

concentrations over six different target protein surfaces at one time. To test this application and illustrate the benefits of this approach, we immobilized the enzyme carbonic anhydrase at different densities (3100–9150 RU) in five channels and left one channel unmodified to serve as a control. Fig. 4 shows the binding responses for six concentrations (0.2–50 μM in a threefold dilution series) of the small molecule inhibitor 4-carboxybenzenesulfonamide (MW = 201 Da, compound D in Table 1) injected for 1 min over all of the surfaces, with the surfaces then washed with running buffer for 2 min. It is important to note that all 36 sensorgrams shown in Fig. 4 were collected simultaneously, with the total time required to collect this entire set of binding responses being less than 4 min. Also, not shown in the figure are the additional 36 sensorgrams from the reference spots between each reaction spot that were collected at the same time. Data from these reference spots were used to correct the reaction surface data for bulk refractive index changes and any systematic baseline drift.

The responses overlaid in each of the plots in Fig. 4 were obtained for six different concentrations of analyte injected across the six target surfaces of different densities. We note that at the highest analyte concentrations, (i) the responses from each surface reach a plateau and (ii) the maximum response level observed is consistent with the fact that we have immobilized decreasing levels of target protein in channels 1 to 5. We also see that there was no binding response observed in the reference channel (6), which did not contain any immobilized protein.

The response data from each surface were globally fit to a simple 1:1 interaction model to determine the association and dissociation rates for the inhibitor/enzyme complex. For each surface, the modeled data (red lines) overlay the experimental data (black lines), indicating that these binding events are well described by a simple interaction model (Fig. 4). The ability to globally fit the concentration series collected from different channels is the first step toward validating the approach of one-shot kinetics.

Kinetic analysis of sulfonamide inhibitors

We used the one-shot kinetics approach to collect binding data for nine sulfonamide-based inhibitors (Table 1) interacting with carbonic anhydrase. For clarity, Fig. 5 depicts the responses collected for each of the compounds from only the highest density surface. In fact, similar data sets were collected for each compound over the five different density carbonic anhydrase surfaces, as illustrated in Fig. 4 for 4-carboxybenzenesulfonamide. In viewing the response data for the nine compounds, we see that there are observable differences in their kinetics. For instance, it is easy to see that (±)-sulpiride (Fig. 5A) has the fastest dissociation rate of the group. We also see that we could detect the binding of very low-molecular mass compounds such as benzenesulfonamide, which has a molecular mass of only 157 Da (Fig. 5G). Each of the data sets was well described by a 1:1 interaction model (for overlays of modeled and experimental data, see Fig. 5). To assess experimental variability, each injection

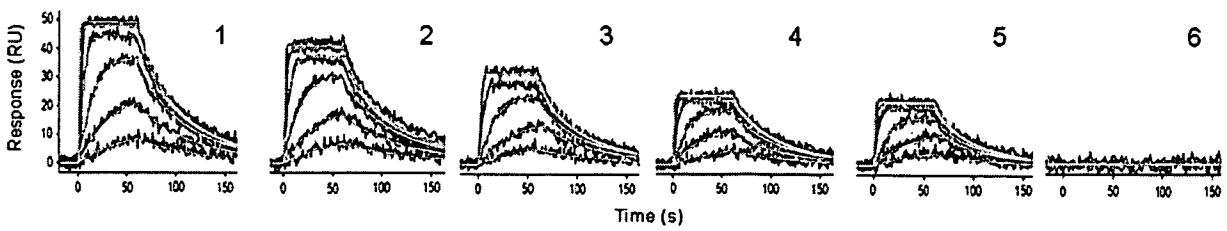

Fig. 4. One-shot kinetic experiment. Binding responses were collected for six concentrations of 4-carboxybenzenesulfonamide (0.2–50 μM in a threefold dilution series) over five different densities of immobilized carbonic anhydrase and a blank surface (labeled 1 to 6). Note that the two highest concentrations nearly overlap because the surface is close to saturation. The flow rate was 100 μl/min, and the association and dissociation times were 1 and 2 min, respectively. Red lines represent a global fit of a simple interaction model to the experimental data (black lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

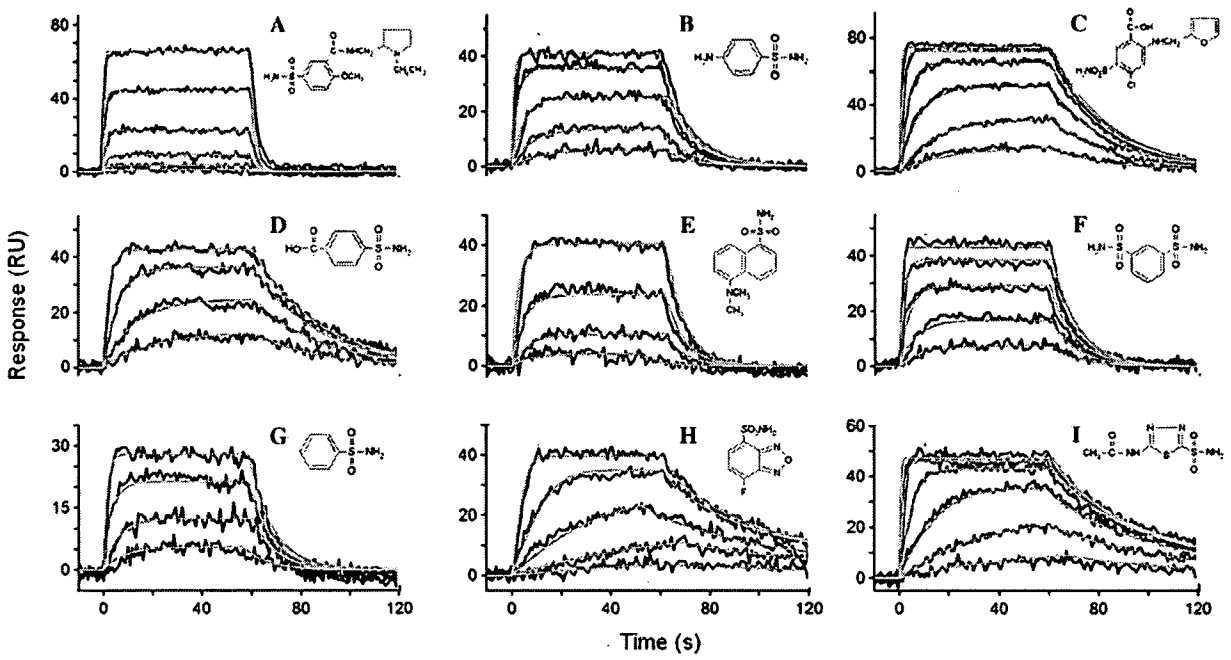

Fig. 5. Kinetic data sets collected for nine inhibitors (compounds A to I in Table 1; structures shown in insets) of carbonic anhydrase. Data from channel 1 only are shown. Red lines represent a global fit of the data to a 1:1 interaction model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

series was repeated twice over the same sensor chip, and then the entire assay was repeated using carbonic anhydrase surfaces prepared in two different ProteOn instruments. In total, this produced 20 kinetic data sets for each compound, and these were analyzed independently to determine the binding constants for the reactions.

The results of the kinetic analysis of all nine compounds are provided in Table 2 and are presented as a plot of association rate constants versus dissociation rate constants in Fig. 6. From the k_a versus k_d plot in the figure, we see both the experimental spread in the rate constants for each compound determined from the 20 replicate studies and how the nine compounds interact differently with the enzyme. The standard errors in the rate constants (k_a and k_d) and equilibrium dissociation constant (K_D) averaged approximately 30 and 19%, respectively. The difference in affinity from the weakest binding compound, (±)-sulpiride (A), to the tightest binding compound, acetazolamide (I) in this data set was approximately 1000-fold, with five compounds

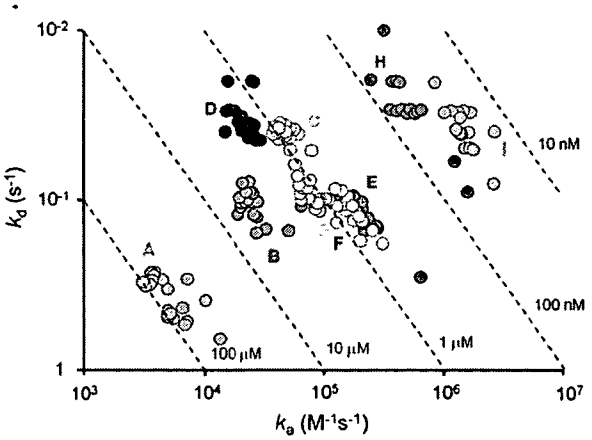

Fig. 6. Association rate constant versus dissociation rate constant plot for nine sulfonamide inhibitors (identified in Table 1) interacting with carbonic anhydrase. The results of 20 replicate analyses are shown for each compound. Isoaffinity lines are shown as dashed diagonals.

Table 2
Binding constants determined at 25 °C from ProteOn XPR36

Sample	Compound	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (µM)
A	(±)-Sulpiride	5.5 ± 2.5E + 3	0.39 ± 0.11	76 ± 18
B	Sulfanilamide	2.8 ± 1.1E + 4	0.11 ± 0.02	4.2 ± 0.9
C	Furosemide	4.9 ± 1.0E + 4	0.04 ± 0.01	0.8 ± 0.1
D	4-Carboxybenzenesulfonamide	1.9 ± 0.8E + 4	0.03 ± 0.01	1.6 ± 0.4
E	Dansylamide	2.2 ± 1.1E + 5	0.13 ± 0.04	0.8 ± 0.3
F	1,3-Benzenedisulfonamide	1.9 ± 0.6E + 5	0.12 ± 0.03	0.64 ± 0.07
G	Benzenesulfonamide	8.5 ± 2.0E + 4	0.10 ± 0.02	1.1 ± 0.1
H	7-Fluoro-2,1,3-benzoxadiazole-4-sulfonamide	5.5 ± 3.2E + 5	0.03 ± 0.02	0.058 ± 0.008
I	Acetazolamide	1.5 ± 0.4E + 6	0.04 ± 0.01	0.025 ± 0.004

(C, D, E, F, and G) clustering around the 1-μM isoaffinity line. Compound A clearly displayed the fastest dissociation rate (~0.4 s^{-1}); compounds B, E, F, and G had intermediate dissociation rates (~0.1 s^{-1}), and compounds C, D, H, and I displayed the slowest dissociation rates (~0.03 s^{-1}).

Surface binding constants versus solution binding constants

To further validate the results from the ProteOn instrument, we compared the affinities determined using the surface-based method with those measured in solution using isothermal titration calorimetry [7,8]. Fig. 7 shows that the correlation in the affinity determined by the two methods was very high with a correlation coefficient of 0.9995. This indicates that immobilization of the enzyme onto the ProteOn sensor surface did not affect its binding affinity for these sulfonamide-based inhibitors. The ability to extract accurate binding constants using the ProteOn instrument also further validates the approach of one-shot kinetics.

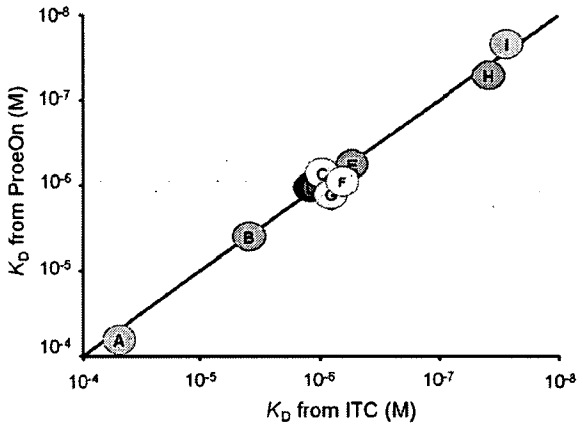

Fig. 7. Correlation plot of the affinity determined by isothermal titration calorimetry (ITC) and by the ProteOn XPR36. Compounds A to I are identified in Table 1. A correlation of 1 is indicated by the diagonal line.

Temperature-dependent studies

To determine how temperature affects the binding constants for these inhibitor/enzyme interactions, we collected response data for four compounds (A, B, D, and F) from 15 to 35 °C in 5 °C increments. Replicate injections of the six analyte concentrations were performed at each temperature and are shown in an overlay plot for each compound in Fig. 8. The data shown in the figure represent a total of 1200 individual responses that were generated using only 40 injections. Apart from the responses obtained for compound F at 15 °C, the data sets appear to be highly reproducible. It is also important to note that we did not observe any spikes in the responses at elevated temperatures that can occur due to outgassing of the buffer. It is likely that the online degassing system built into the ProteOn system helps to reduce outgassing.

When visually comparing the dissociation phase data in each column in Fig. 8, it is clear that the dissociation rate for each compound increases with temperature, as would be expected. To compare the temperature effect more rigorously, the data sets for each compound at a given temperature were globally fit to a 1:1 interaction model. In this case, we globally fit all of the data from the different density surfaces with one association and dissociation rate constant while allowing a local parameter for the surface capacity

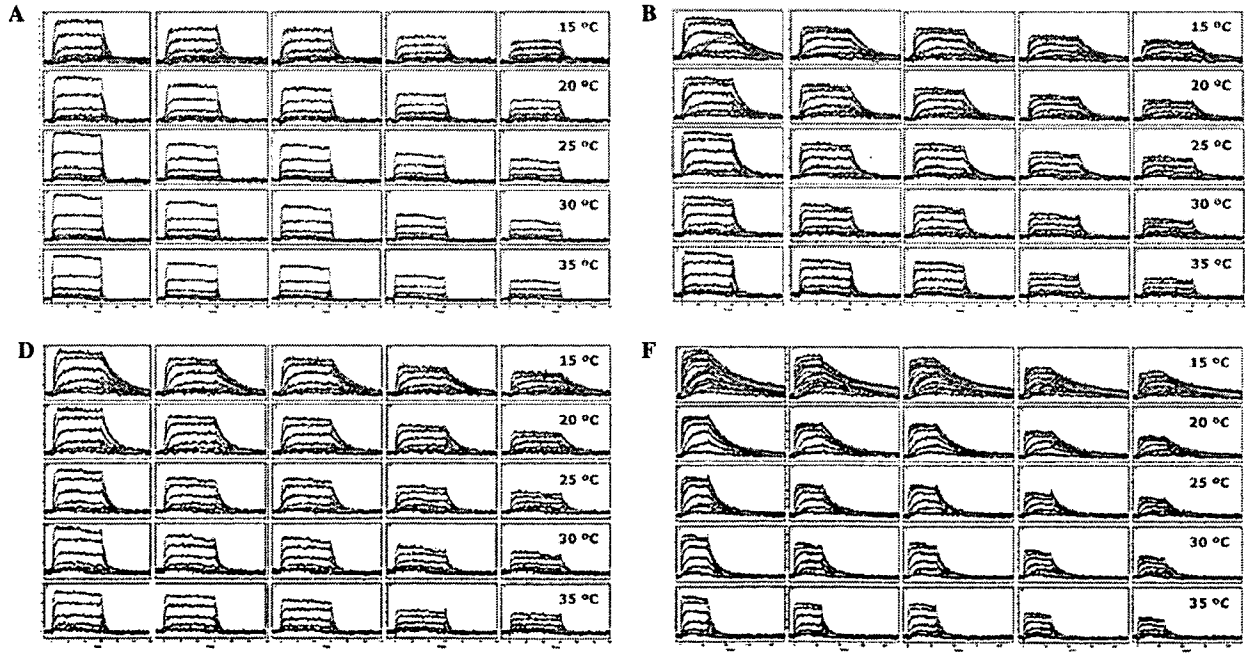

Fig. 8. Temperature-dependent binding data for compounds A, B, D, and F. Six concentrations of each compound were tested in duplicate for binding to five different densities of immobilized carbonic anhydrase. Compounds were tested from 15 to 35 °C in 5 °C increments as labeled.

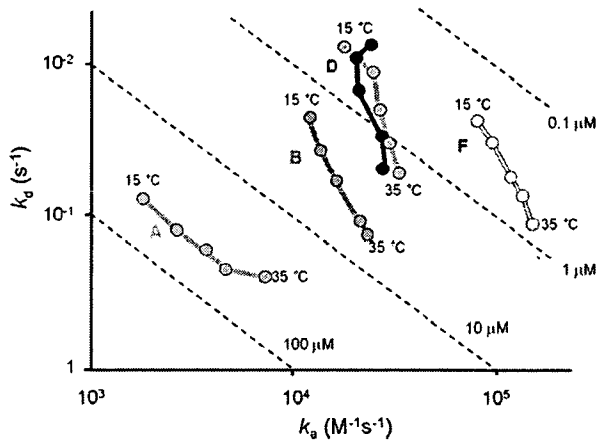

Fig. 9. Temperature dependence of the binding constants for four sulfonamide inhibitors of carbonic anhydrase. Compounds A, B, D, and F were tested at temperatures of 15, 20, 25, 30, and 35 °C. Compound D was tested twice, as shown by the light and dark blue lines and symbols. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(R_{max}). As illustrated by the overlay of the modeled and experimental data, the responses from different density surfaces could be fit well using this global approach (Fig. 8). The temperature dependence of the binding constants for each compound is shown graphically in Fig. 9. Compound D was actually run twice to assess overall experimental variability, which was found to be very good. In general, each compound shows an increase in the association and dissociation rates with increasing temperature, and this is fairly typical of molecular interactions. To a large extent, these changes in the rate constants offset one another, so that the overall change in affinity over the 20 °C change in temperature averages approximately fourfold for each compound.

Discussion

The crisscrossing flow paths in Bio-Rad's new ProteOn XPR36 array biosensor offers a novel approach toward kinetic analysis of molecular interactions. Perhaps most interesting is the concept of one-shot kinetics, which takes advantage of two key aspects of the ProteOn technology. First is the ability to deposit a uniform distribution of a target molecule across six channels on the sensor surface. We found that the variation in immobilization density was less than 3% for carbonic anhydrase. Second is the ability to switch the direction of the flow channels to create a 36-spot array. In the one-shot kinetic format, it is possible to test six different concentrations of the analyte across these spots at once to collect concentration-dependent binding data. Historically, the data from an analyte concentration series were collected serially, with the biggest challenge coming from the need to regenerate the binding surface between binding cycles. The one-shot approach allows one to collect concentration-dependent responses across independent reaction spots, thereby eliminating the need to regenerate the surface. Most commercial SPR instruments that are in the field are not capable of performing simultaneous injections of analyte.

To test the approach of one-shot kinetics and the sensitivity of the ProteOn system for small molecule analysis, we

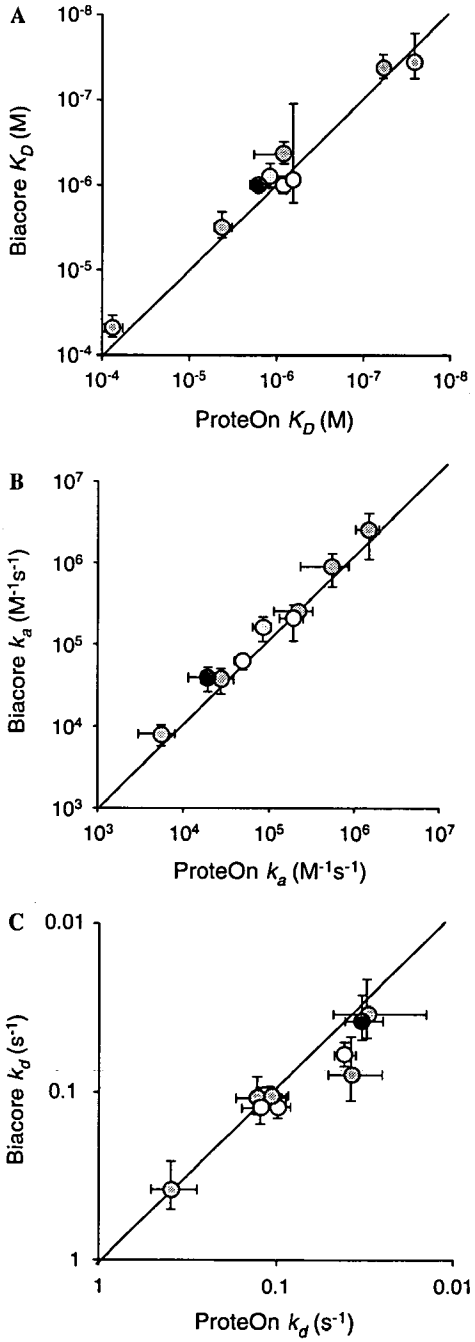

Fig. 10. Comparison of binding constants determined from ProteOn XPR36 and Biacore platforms. Plots A, B, and C show the comparison of the equilibrium dissociation constants, association rate constants, and dissociation rate constants, respectively. The error bars on the x axis represent standard errors determined from an analysis of 20 replicate data sets from the ProteOn platform. The values and standard errors on the y axis were determined from a benchmark study using different Biacore instruments as described in Ref. [8].

ran a series of experiments with a well-characterized system, namely the binding of sulfonamide-based inhibitors to carbonic anhydrase [7–10]. By coupling the enzyme at different densities in the five channels and leaving one channel blank, we were able to confirm that the binding responses for the small molecule analytes correlated with the amount of enzyme immobilized. Importantly, we found that it was possible to globally fit the response data for all nine of the test compounds over all of the reaction surface spots using a simple interaction model. These results validate the one-shot approach for kinetic analysis. We also showed that the affinities determined for the nine compounds from the ProteOn system correlated with the affinities determined in solution using titration calorimetry. These results demonstrate that in this case immobilization of the enzyme to the surface did not change its binding properties. Finally, the binding constants determined from the ProteOn system correlated with the results obtained from Biacore. The data in Fig. 10 show a visual comparison of the affinities as well as association and dissociation rate constants for these same analytes determined from a recent benchmark study we coordinated on Biacore instruments [8].

The ability to collect response data in real time for six different concentrations of analyte across six different surfaces is a significant advancement over serial processing biosensor systems [11]. Even for complexes that do not need regeneration, the ability to parallel process six samples increases throughput by a minimum of sixfold. Our carbonic anhydrase experiments used a cycle time of approximately 6 min/injection, making it possible to test one 96-well plate of samples in approximately 90 min or 16 plates in 24 h with a total sample throughput of more than 1500 individual analytes. When one considers that six samples may be tested against six different target proteins, the overall improvement in throughput can be significant. But perhaps the most significant advancement comes from the ability to collect response data for six analyte concentrations simultaneously without the need to regenerate the surface. We have found that regeneration has become a serious bottleneck in throughput, particularly for small molecule interactions with high affinity ($K_D < 100$ nM).

Of course, it should be noted that our use of five different surface densities of the target protein is not required to characterize an interaction. We used multiple surface densities to show that the kinetics from the different reaction surfaces could be globally fit and were independent of surface density (as expected). In practical applications, the six different channels could be used to screen compounds against six different target proteins. Also, it should be pointed out that the ProteOn system allows the fluidic channels to be rotated back and forth over the same chip surface. This makes it possible to perform six different immobilizations, and this would prove to be invaluable if one wanted to use a single chip to characterize six analytes that are difficult to regenerate.

In terms of both immobilization strategies and analyte testing, there clearly are a number of unique applications for the ProteOn's crisscrossing flow system. As we have illustrated here, the introduction of one-shot kinetics is a significant advancement in the approach to biosensor analysis that could have an immediate impact on a large number of biosensor users.

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References

[1] R.L. Rich, D.G. Myszka, Survey of the year 2004 commercial optical biosensor literature, J. Mol. Recogn. 18 (2005) 431–478.
[2] I. Navratilova, D.G. Myszka, Applications of SPR biosensors, in: J. Homola (Ed.), Springer Series on Chemical Sensors and Biosensors, in press.
[3] R.L. Rich, D.G. Myszka, Why you should be using more SPR biosensor technology, Drug Discov. Today 1 (2004) 301–308.
[4] S.D. Long, D.G. Myszka, Affinity-based optical biosensors, in: D.G. Hage, J. Cazes (Eds.), Handbook of Affinity Chromatography, CRC Press, Boca Raton, FL, 2005, pp. 685–698.
[5] R.L. Rich, Y.S.N. Day, T.A. Morton, D.G. Myszka, High-resolution and high-throughput protocols for measuring drug/human serum albumin interactions using Biacore, Anal. Biochem. 296 (2001) 197–207.
[6] T.A. Morton, D.G. Myszka, Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors, Methods Enzymol. 295 (1998) 268–295.
[7] Y.S.N. Day, C.L. Baird, R.L. Rich, D.G. Myszka, Direct comparison of equilibrium, thermodynamic, and kinetic rate constants determined by surface- and solution-based biophysical methods, Protein Sci. 11 (2002) 1017–1025.
[8] G.A. Papalia, et al., Comparative analysis of ten small molecules binding to carbonic anhydrase II by different investigators using Biacore technology, Anal. Biochem., submitted for publication.
[9] M.J. Cannon, et al., Comparative analyses of a small molecule/enzyme interaction by multiple users of Biacore technology, Anal. Biochem. 330 (2004) 98–113.
[10] D.G. Myszka, Y.N. Abdiche, F. Arisaka, O. Byron, E. Eisenstein, P. Hensley, J.A. Thomson, C.R. Lombardo, F. Schwarz, W. Stafford, M.L. Doyle, The ABRF–MIRG '02 study: Assembly state, thermodynamic, and kinetic analysis of an enzyme/inhibitor interaction, J. Biomol. Tech. 14 (2003) 247–269.
[11] D. Reichmann, O. Rahat, S. Albeck, R. Meged, O. Dym, G. Schreiber, The modular architecture of protein–protein binding interfaces, Proc. Natl. Acad. Sci. USA 102 (2004) 57–62.